

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 August 2003 (07.08.2003)

PCT

(10) International Publication Number
WO 03/064627 A2

(51) International Patent Classification: C12N

(21) International Application Number: PCT/US03/03235

(22) International Filing Date: 3 February 2003 (03.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/067,148 1 February 2002 (01.02.2002) US

(71) Applicant: ORIGEN THERAPEUTICS [US/US]; 1450
Rollins Road, Burlingame, CA 94010-2307 (US).

(72) Inventors: ETCHES, Robert, J.; 111 East 9th Avenue,
Apt. 103, San Mateo, CA 94401 (US). VAN DE LAVOIR,
Marie-Cecile; 22 Gaviota Way, San Francisco, CA 94127
(US). HEYER, Babette; 806 Coleman Avenue, #5, Menlo
Park, CA 95025 (US). DIAMOND, Jennifer; 421 Elder
Avenue, Millbrae, CA 94030 (US). MATHER, Christine;
1410 Millbrae Avenue, #203, Millbrae, CA 94030 (US).

BEEMER, Kathleen; 654 Hickey Blvd., Pacifica, CA
94044 (US). MYERS, Heather; 191 Sonora Avenue,
Danville, CA 94526 (US).

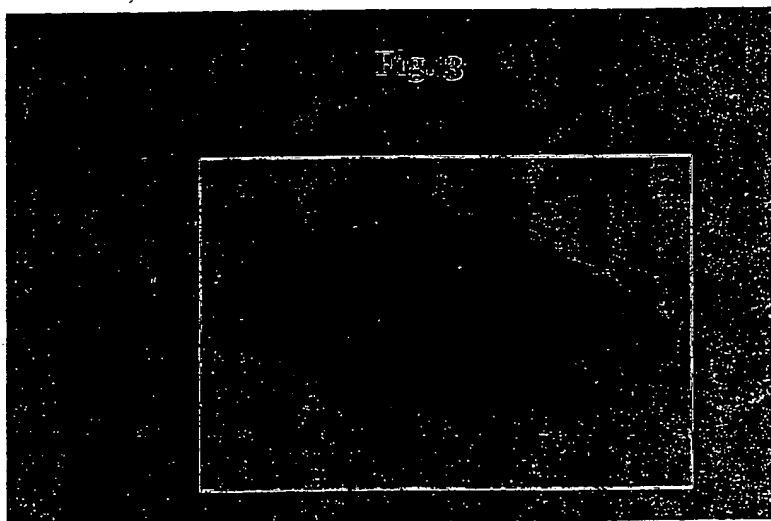
(74) Agents: MULVILLE, Kurt et al.; Orrick Herrington &
Sutcliffe LLP, 4 Park Plaza, Suite 1600, Irvine, CA 92614-
2558 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC,
VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: CHIMERIC BIRD FROM EMBRYONIC STEM CELLS



(57) Abstract: Sustained cultures of avian embryonic stem cells are provided. Injection of avian embryonic stem cells into recipient embryos yields chimeras with a significant contribution from the embryonic stem cell phenotype. Transgene encoding exogenous proteins are stably integrated in the embryonic stem cells and are present in the somatic tissue of the resulting chimeras. The transgenes may encode exogenous proteins expressed in endodermal, ectodermal, mesodermal, or extra embryonic tissue. Breeding the resulting chimera yields transgenic birds whose genome is comprised of exogenous DNA.



WO 03/064627 A2

**Declarations under Rule 4.17:**

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for all designations*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

- *without international search report and to be republished upon receipt of that report*

CHIMERIC BIRD FROM EMBRYONIC STEM CELLS

FIELD OF THE INVENTION

This invention relates to the field of transgenic and chimeric animals and avian embryonic stem cells sustained in long-term cultures to produce chimeras when injected into
5 recipient embryos. The cells may contain engineered genetic constructs that introduce genetic modification into birds, including transgenes encoding exogenous proteins that may be expressed in the somatic tissue.

BACKGROUND OF THE INVENTION

10 Gene engineering of animals can create valuable animals that can produce pharmaceutical products in their cells. However, incorporating genetic modifications into an animal's permanent DNA is a major technological hurdle. Because embryonic stem cells contribute to the permanent DNA of an animal, the characteristics of an embryonic stem cell donor animal may be transferred by incorporating these cells into a recipient animal in an
15 embryonic state. This offers two advantages: the phenotype of the donor animal can be selectively transferred to a recipient embryo. Second, uniquely stable embryonic stem cell cultures can be genetically modified to introduce modifications into the recipient embryo.

The embryonic stem cells can be engineered with a transgene that encodes an exogenous protein and incorporated into genetically engineered animals to produce exogenous
20 proteins, particularly when tissue specific expression is obtained.

A transgene integrated in a sufficiently stable embryonic stem cell culture will encode the protein in a new chimeric or transgenic organism. Depending on the specific transgene, whole genomes can be transferred by cell hybridization, intact chromosomes by microcells, subchromosomal segments by chromosome mediated gene transfer, and DNA fragments in the
25 kilobase range by DNA mediated gene transfer (Klobutcher, L.A. and F.H. Ruddle, Annu. Rev. Biochem., 50: 533-554, 1981). Intact chromosomes may be transferred by microcell-mediated chromosome transfer (MMCT) (Fournier, R.E. and F.H. Ruddle, Proc. Natl. Acad. Sci. U.S.A., 74: 319-323, 1977).

Genetic modifications of embryonic stem cells to produce transgenic animals has been
30 demonstrated in only a very few species. In mice, homologous recombination followed by chromosome transfer to embryonic stem (ES) cells produces chimeric and transgenic offspring. In murine ES cells, site-specific homologous recombination and gene targeting have been developed, (Thomas, Capecchi, Cell 1987) insertion of cloned DNA and manipulation of chromosome fragments by the Cre-loxP system techniques (Smith, et al., Nat. Genet. 1995)
35 produce stable genetic chimeras. Unfortunately, techniques that are useful in mammalian

systems may not be available in non-mammalian systems because of the absence of the necessary long-term ES cell cultures.

Embryonic stem cell lines suitable for use in transgenesis must be stable and maintain pluripotency when the ES cell is transfected with a genetic construct, when the cell is selected
5 for transformation, and when injected embryos to form chimeras. Ideally, the ES cells incorporate transgene and the resulting chimeric animal would selectively express the transgene in selected tissue types. For example, transgenes encoding DNA derived from the lymphoid elements of the immune system might be targeted to be expressed in B lymphocytes of a chimeric or transgenic animal. To achieve this, the ES cell culture must facilitate
10 transformation with DNA encoding an exogenous protein, and the ES cell must contribute significantly to the genome of the resulting animal.

Avian biological systems offer many advantages for exogenous protein expression, including efficient farm cultivation, rapid growth, and economical production. Globally, chickens and turkeys are grown in larger numbers for the human diet. Further, the avian egg
15 offers an ideal biological design, both for massive synthesis of a few proteins and ease of isolation of protein product. However, application of the full range of mammalian transgenic techniques to avian species has been unsuccessful. Most notably, the transmission to a mature, living animal of a genetic modification introduced into an avian ES cell has not been demonstrated.

To achieve the success already seen in mice, avian transgenesis requires long-term
20 avian ES cells cultures that maintain pluripotency. Techniques to introduce genetic modifications into ES cells, screening, selection, and manipulation for injection into embryos requires several weeks for all of the steps. Thus, to be useful in transgenesis, pluripotency must be consistently maintained until embryo injection and the ES cell must be incorporated
25 into a recipient embryo to a substantial degree.

In the absence of ideal ES cell culture conditions, ES cells rapidly differentiate and lose the ability to contribute to the somatic tissue of a chimera. Current avian ES cell culture techniques limit the period of pluripotency and desirable chimeric or transgenic avians, expressing exogenous proteins cannot be created.

SUMMARY OF INVENTION

Long-term avian ES cell cultures and special techniques produce chimeric and transgenic birds derived from prolonged ES cell cultures. In some embodiments, pluripotent ES cells are transfected to modify the DNA of the ES cell with a transgene encoding an exogenous protein. Injection into a host avian embryo and transgenic birds exhibit an ES-cell derived phenotype and express a selected foreign protein in accord with the regulatory elements incorporated into the transgene. This invention includes compositions comprising long-term cultures of chicken ES cells that can be genetically modified to contribute to the somatic tissues of recipient embryos.

DESCRIPTION OF THE FIGURES

Figure 1 shows the characteristic morphology of chicken ES cells where the small cells grow in a single layer with little cytoplasm and a prominent nucleolus.

Figure 2 is a karyotype of chicken ES cells that have been in culture for 189 days. The cells are diploid, carry 38 pairs of autosomal chromosomes and one pair of Z chromosomes.

Figure 3 is two Barred Rock chicks and two chimeras formed by injecting Barred Rock ES cells into a White Leghorn recipient embryo. The chimeras and the Barred Rocks are indistinguishable indicating that the contributions of the ES cells to the melanocyte lineage is extensive.

Figure 4 are chimeras made by injecting Barred Rock ES cells into White Leghorn recipients. The pair of chimeras in the left panel exhibit minor contributions to the melanocyte lineage whereas the pair in the right panel show more extensive contributions.

Figure 5 is a diagram of the pCX/GFP/Puro plasmid construct used for transfection of ES cells.

Figure 6 is a FACS analysis of non-transfected chicken ES cells (upper panel) and chicken ES cells that have been transfected with the pCX/GFP/Puro construct and grown in the presence of puromycin. The analysis shows that substantially all of the transfected cells are expressing the transgene.

Figure 7 is a Southern analysis of ES cells that have been transfected with the pCX/GFP/Puro construct. The difference in the location of the probe in preparations of DNA digested with BamH1, EcoR1 and a combination of the two endonucleases indicates that the transgene is incorporated into the genome at different sites in the cell lines TB01 and TB09.

Figures 8A and 8B: (A) A diagrammatic representation of a transgene designated Ov7.5MAbdns (upper panel); (B) A diagrammatic representation of a transgene designated Ov15MAbdns (lower panel).

Figure 9 is the genomic PCR analysis of BAC-A transfected cES cells.

5 Figure 10 is a Southern analysis of chimeras made with cES cells transfected with CX/GFP/Puro.

Figure 11 shows the correlation between estimates of chimerism derived by scoring the level of fluorescence in skin and comb tissues and derived by scoring the extent of black pigmentation of the chick at hatch.

10 Figure 12 is a summary of FACS data from chimeras made with non-transfected cells (control), chimeras made with ES cells that were transfected with CX/GFP/Puro and estimated to contain <1-5% donor-derived cells by feather pigmentation, chimeras made with ES cells that were transfected with CX/GFP/Puro and estimated to contain >5% and <10% donor-derived cells by feather pigmentation, chimeras made with ES cells that were transfected with
15 CX/GFP/Puro and estimated to contain >10% and <30% donor-derived cells by feather pigmentation, and chimeras made with ES cells that were transfected with CX/GFP/Puro and estimated to contain approximately 75% donor-derived cells by feather pigmentation. Cells were prepared as described in the text from liver, brain, and muscle. The mean number of fluorescent cells detected above the autofluorescent threshold is shown in the left panel; the
20 mean number of fluorescent cells detected above the M2 threshold, which is an order of magnitude higher than the autofluorescent threshold is shown in the right panel.

Figure 13 is an example of a FACS analysis of cells prepared from the brain of a chimera made with non-transfected ES cells (upper panel) and chimeras that were made with cells transfected with CX/GFP/Puro (lower panel). Substantially all of the cells from the
25 chimera made with transfected cells display a level of fluorescence that is above that of the cells from the control bird indicating that the contribution of the donor ES cells to the brain tissue of the chimera is extensive.

Figure 14 is an example of a FACS analysis of cells prepared from leg muscles of a chimera that was made with cells transfected with CX/GFP/Puro. The right leg of this chimera
30 was green to the naked eye indicating substantial contribution to this tissue. A preparation of cells from the fluorescent leg muscle was shown to contain primarily fluorescing cells by FACS analysis (lower panel). The left leg of the chimera was normal in color and a preparation of these cells was shown to contain non-fluorescing cells by FACS analysis. These data show that chimerism in tissues within the same animal may receive different contributions
35 from donor ES cells.

DETAILED DESCRIPTION OF INVENTION

Stable, long-term cultures of ES cells that yield genetically engineered chickens are necessary for avian transgenics, including specifically protein production for the pharmaceutical industry, production of chickens that deposit human antibodies in their eggs, and site-specific modification of the avian genome for other applications. Chimeric or transgenic avians require chicken ES (cES) lines that contribute to somatic tissues when injected into a recipient embryo. Long-term cES cultures also enable selection of desirable phenotypes in chimeric animals and modifications to the genome of cES cell to introduce exogenous DNA into a chimeric offspring. In preferred embodiments, extended avian cES cultures are engineered to contain transgene derived DNA to produce chimeras containing transgene-derived DNA in the somatic tissues. In particularly preferred embodiments, exogenous proteins encoded by the transgene are expressed in all tissue types, or in tissue types selected according to the regulatory elements encoded by the transgene.

In this invention, cES cell lines derived from stage X embryos have a large nucleus and contain a prominent nucleolus (Figure 1) and are confirmed to be cES cells by morphology after long-term culturing and yield chimeras when injected into recipient embryos. The cES cells contribute to somatic tissues in a high degree as determined by extensive feather chimerism. Still further, these cES cells can be transfected with transgenes carrying DNA encoding an exogenous protein. The ES cells stably integrate the transgene, express the transgene for selection, and form chimeras that may express the exogenous protein in somatic tissue of the chimera or may express the transgene in selected somatic tissue. As used herein, the description of cES cell progeny are those that differentiate into non-ES cell phenotypes after introduction of the ES cells into recipient embryos and the formation of a chimera. Expression of the transgene in somatic tissue is demonstrated in extraembryonic and somatic tissues including the allantois, endoderm, mesoderm, and ectoderm of the chimeric animal and may be broadly expressed in all tissues and organ types.

Example 1 - Derivation of chicken embryonic stem cells (cES cells)

Chicken ES cells were derived from one of two crosses: Barred Rock X Barred Rock or Barred Rock X Rhode Island Red. These breeds yield a feather marker for testing the developmental potential of cES cells. The cES cells are injected into White Leghorn embryos, which are homozygous dominant at the dominant, white locus. Chimeric chickens resulting from injection display black feathers from the donor cES cells and white feathers from the recipient embryo.

The cES cell culture was initially established according to J. Petitte USP 5,565,479. At stage X, the embryo is a small round disk, consisting of approximately 40,000-60,000 cells, situated on the surface of the yolk. To retrieve the embryo, a paper ring is put on the yolk membrane, exposing the embryo in the middle. The yolk membrane is cut around the ring, which is then lifted off the yolk. The embryo, attached to the ventral side of the ring, is placed under the microscope and the area pellucida isolated from the area opaca using a fine loop.

Table 1: cES cell lines derived on either STO feeder cells or a polyester insert in CES-80 medium initiated from either single or pooled embryos.

Cell line	Donor embryo	Substrate used to derive cES cells	Endpoint of cell line
009	pooled	STO	cultured for 3 months, injected & cryopreserved
029	pooled	insert	cultured for over 3 months, injected & cryopreserved
31	pooled	STO	injected at 4 days
36	pooled	STO	injected at 13 days
50	pooled	STO	grown for over 8 months, injected & cryopreserved
63b	pooled	insert	grown for 3 months and cryopreserved
67I	single	insert	injected at 45 days of culture
307	pooled	STO	injected at 15 days and fixed for staining
314	pooled	STO	cultured for over 3 months, injected & cryopreserved
317	pooled	STO	injected at 12 days and fixed for staining
324A	single	insert	cultured for over 6 months and injected
328	single	insert	cultured for over 6 months, injected & cryopreserved
329	single	insert	cultured for 5 months, injected & cryopreserved
330	single	insert	cultured for 3 months and cryopreserved
331	single	24 w insert	cultured for over 3 months and terminated
332	single	96 w STO	cultured for 3 months and cryopreserved
333	single	12 w insert	cultured for over 3 months and terminated
334	single	12 w insert	cultured for over 3 months and terminated
335	single	96 w insert	cultured for over 3 months and terminated

Embryos are dispersed mechanically into a single cell suspension and seeded on a confluent feeder layer of mitotically inactivated STO cells at a concentration of 3×10^4 cells/cm². The cES culture medium consists of DMEM (20% fresh medium and 80% conditioned medium) supplemented with 10% FCS, 1% pen/strep; 2mM glutamine, 1mM pyruvate, 1X nucleosides, 1X non-essential amino acids and 0.1mM β -mercaptoethanol. Before use, the DMEM medium is conditioned on Buffalo Rat Liver (BRL) cells. Briefly, after BRL cells are grown to confluency, DMEM containing 5% serum is added and conditioned for three days. The medium is removed and a new batch of medium conditioned for three days and repeated. The three batches are combined and used to make the cES medium. Chicken ES

cells become visible 3-7 days after seeding of the blastodermal cells. These cES cells were morphologically similar to mES cells; the cells were small with a large nucleus and a pronounced nucleolus (See Figure 1).

The growth characteristics of cES cells are different from murine (mES) cells, which grow in tight round colonies with smooth edges and individual cells that are difficult to distinguish. cES cells grow in single layer colonies with clearly visible individual cells. Tight colonies are often the first sign of differentiation in a cES culture.

To test for markers of pluripotency of the cells that were derived in culture, the cells were fixed and stained with SSEA-1 (Solter, D. et al., PNAS 1978), EMA-1, which recognize epitopes on primordial germ cells in mice and chickens (Hahnel, Eddy, Gamete Research 1986) and alkaline phosphatase (AP) which is also expressed by pluripotential cells. The results of these tests demonstrate that chicken ES cells express alkaline phosphatase and the antigens recognized by SSEA-1 and EMA-1.

Although cES cells are visible using the above protocol, such cultures cannot be maintained longer than a few weeks. Several modifications in culture conditions yield the cell lines (Table 1) of which 14 were tested for pluripotency by injection into recipient embryos. Eleven of the 14 cell lines contributed to recipient embryos as determined by feather pigmentation (See Table 2 below). This protocol yields sustained cultures of pluripotent cells expressing an ES cell phenotype. At any point, the cells can be cryopreserved and when injected into compromised recipient embryos have the potential to substantially contribute to somatic tissues (see Examples 3 and 5 below) even when maintained as culture for 60, 180, 360 days or longer.

Table 2: Passage number and time in culture of embryonic stem cell lines derived from single or pooled embryos. Frequency and extent of somatic chimerism after injection of these cES cells into stage X recipients.

Cell line	Donor embryo	Passage number	time in culture (days)	# of embryos injected	# chimeras	# analyzed	% chimeras	Extent of chimerism ¹ (%)
31	pooled	0	4	15	2	7	28.5	1-5
317	pooled	4	12	29	2	10	20	25-65
36	Pooled	1	13	24	1	5	20	15
307	pooled	4	15	21	1	6	17	5
330	single	6	33	11	3	8	25	5-50
63b	pooled	11	72	36	4	21	19	1-10
67I	single	3	45	28	0	15	0	-

324A	single	10	65	25	0	15	0	-
009	pooled	20	61	27	0	9	0	-
329	single	3	15	31	8	17	47	3-75
329		6	25	30	9	19	47	3-95
329		6	28	26	1	12	8	23
329		11	49	10	1	4	25	60
029	pooled	4	33	40	9	27	33	5-80
029		9	37	40	4	15	27	4-15
328	Single	6	56	19	4	11	36	10-80
328		12	98	33	7	22	32	5-50
314	Pooled	17	52	30	2	5	40	5-65
314		15-17	53	29	1	4	25	30
314		17	55	37	3	15	30	3-80
314		16	65	27	2	11	18	5-40
314		14	61	25	0	13	0	-
314		16	65	32	3	14	21	10-60
314		20	61	30	4	5	80	4-50
314		21	67	30	2	11	18	5-15
314		21	71	8	0	2	0	-
50	pooled	7	53	35	7	23	30	4-65
50		14	106	36	3	21	14	10-30

[†]Extent of chimerism was determined by the proportion of black feathers.

As with the mouse, avian embryonic stem cells, which are sometimes referred to as embryonic germ cells, are derived on a variety of feeder layers including STO, STO-snl and others that are readily available. In a preferred embodiment, cES cell cultures are initiated on a STO feeder layer. STO cells are grown to confluency, treated with 10µg/ml mitomycin for 3-4 hours, washed, trypsinized and seeded on gelatin coated dishes at 4×10^4 cells/cm². cES cells appear to grow more rapidly when the feeder of STO cells are sparser. Reducing the STO feeder cell concentration to between 10^3 and 10^5 , and preferably below 10^4 cells/cm², facilitates the derivation and propagation of cES cells. However, when chicken embryonic fibroblasts and mouse primary fibroblasts are used as feeders, no cES cells were derived. Also, when previously established cES cells were plated on these feeders, all of them differentiated within 1 week.

Growing cES cells on synthetic inserts, such as polymer membranes (Costar, Transwell type) without feeders avoids contamination of the recipient embryo with feeder cells when the ES cells are injected. As Table 3 and 4 show, culturing on inserts, instead of STO feeders, facilitates the derivation of cES cells, and inserts can be used for initial derivation. However, after initially growing rapidly on inserts, the mitotic activity of the ES cells declines after 4-6 weeks of culture. To extend the culture the cells have to be transferred to a feeder of STO cells.

Table 3. Establishment of cES cells from single embryos on either inserts or a feeder of STO cells (10^4 cells/cm²).

Substrate	# of cultures started	# of cell lines obtained
STO feeder	56	3 (5%)
insert	45	7 (16%)

5 Table 4. Establishment of cES cells from pooled embryos on either a STO feeder or a synthetic insert.

Substrate	# of cultures started	# of cell lines obtained
STO feeder	73	7 (9.5%)
insert	17	2 (12%)

10 The data in Tables 3 and 4 show that chicken embryonic feeder cells and mouse primary fetal fibroblasts do not support cES cells. A feeder of STO cells supports derivation and growth but only in a limited concentration of between 10^3 and 10^5 STO cells, but preferably in the present embodiment at a concentration of less than or appropriately 10^4 cells/cm². A dense STO feeder layer impairs the growth of cES cells, while the specified
 15 concentration of STO cells provides factor(s) necessary for ES cell proliferation. When the cells are sustained over an extended culture period, continue to express an ES cell phenotype, and differentiate into non-embryonic stem cell phenotypes in vivo, they are referred to as "ES cell progeny."

The cES cell culture medium consists of 80% conditioned medium and preferably
 20 contains certain BRL-conditioned medium with factors necessary for the derivation and growth of cES cells. At a concentration of 50%, growth of the cES cells is not as reliable as in 80% conditioned medium. When the percentage of conditioned medium is reduced to less than 50%, the growth of the cES cells is affected, as evidenced by an increase in differentiated cells and, at a concentration of 30% or less, the cES cells differentiate within 1 week. This
 25 conditioned medium, found necessary for the derivation and maintenance of cES cells, does not maintain mES, but causes their differentiation.

Fetal bovine serum is a preferred component of the ES cell medium and contains factors that keep cES cells in an undifferentiated state. However, serum is also known to contain factors that induce differentiation. Commercially available serum lots are routinely
 30 tested by users for their potential to keep ES cells in an undifferentiated state. Serum used for cES cell cultures are known to differ from serum used for mES cell cultures. For example, serum used for the culture of mES cells that is low in cytotoxin and hemoglobin concentration,

which is known to maintain mouse ES cells in an undifferentiated state, did not support the sustained growth of cES cells. Preferably, cell cultures are divided into two and used to test each new batch of serum. The new batch tested must clearly support the growth of chicken ES cells when empirically tested.

5 Chicken chromosomal spreads require special evaluation techniques different than mice because the complex karyotype consisting of 10 macrochromosomes, 66 micro-chromosomes, and a pair of sex chromosomes (ZZ in males and ZW in females). The long-term cES cells of the invention shown in Figure 2, analyzed after 189 days in culture and after being cyopreserved twice, exhibited a normal karyotype with 10 macro chromosomes; 2 Z-
10 chromosomes and 66 microchromosomes. Chicken ES cells are cryopreserved in 10% DMSO medium. After thawing and injecting several cell lines into recipient embryos, somatic chimeras are obtained, indicating that the cES cells maintain their developmental potential during the cryopreservation process.

15 **Example 2 – Injection of chicken embryonic stem cells into recipient embryos.**

 To permit access to the embryo in a freshly laid egg the shell must be breeched, inevitably leading to a reduction in the hatch rate at the end of the 21-day incubation period. Conventional methods cut a small hole in the side of the egg, through which the embryo is manipulated, and re-seal with tape, a glass cover slip, shell membrane or a piece of shell. The
20 conventional method causes embryonic mortality between 70 and 100%. Improved access to the embryo and increased survival and hatchability is achieved by transferring the embryo to a surrogate eggshell for incubation to hatching using two different shells and the method disclosed in Callebaut, Poult. Sci 60: 723-725 (1981) and Rowlett, K. and K. Simkiss, J. Exp. Biol. 143: 529-536, 1989. With this technique, the mean hatch rate is approximately 41%
25 (range 23-70%) with 191 chicks hatched from 469 cES-cell injected embryos.

 Incubation of embryos following injection of donor ES cells into recipient embryos can be divided into two parts comprising System A and System B as described below:

 System A covers the first three days of post-oviposition development. Fertile eggs containing the recipient embryos are matched with eggs 3 to 5 grams heavier. A 32mm
30 diameter window is cut at the pointed pole, the contents removed and the recipient embryo on the yolk, together with the surrounding albumen, is carefully transferred into the surrogate shell.

 Cells are taken up in a sterile, finely tapered glass pipette connected to a mouth aspirator fitted with a 2 micron filter. The opening of the pipette can be from 50 to 120
35 microns in diameter and possesses a 30° spiked bevel. The embryo is visualized under low

magnification and with blue light. Chicken ES cells are trypsinized into a single cell suspension and 4,000 to 26,000 cells are injected into an embryo. The cells are gently expelled into the space either below or above the embryo, i.e. into the sub-embryonic cavity or between the apical surface of the area pellucida and the perivitelline layer (yolk membrane). Extra
5 albumen collected from fresh fertile eggs is added and the shell sealed with Saran Wrap plastic film.

System B covers the period from day three to hatching. At day three of incubation the embryo has reached around stage 17 (H&H). Water has been transported from the albumen to the sub-embryonic cavity, causing the yolk to enlarge and become very fragile. The contents
10 of the System A shell are very carefully transferred to a second surrogate shell (usually a turkey egg) 30 to 35 grams heavier than the original egg. Penicillin and streptomycin are added to prevent bacterial contamination and the 38 to 42mm window in the blunt pole is sealed with plastic film. This larger shell allows for an artificial airspace. At day 18 to 19 of
15 incubation the embryo cultures are transferred to tabletop hatchers for close observation. As lung ventilation becomes established, holes are periodically made in the plastic film to allow ambient air into the airspace. Approximately 6-12 hours before hatching the film is replaced with a small petri dish, which the chick can easily push aside during hatching.

For incubation, conventional temperature (37.5 to 38°C) and relative humidity (50 to 60%) are maintained for the embryos in surrogate shells, but periodic egg rocking, which is
20 normally hourly and through 90 degrees, has to be modified to ensure good survival. In System A rocking is through 90° every 4 to 5 minutes; in System B it is through 40 to 60° every 40 to 45 minutes. In both systems the speed of rocking is maintained at 15 to 20° per minute.

The contribution of cES cells to chimeras is improved if the recipient embryo is
25 prepared by (1) irradiated by exposure to 660 rads of gamma irradiation (2) altered by mechanically removing approximately 1000 cells from the center of the embryo, or by combining (1) and (2) above before the injection of the cES cells. Referring to Table 5, contribution of cES cells to the somatic tissues increased substantially when recipient embryos were compromised, either by removing cells from the center of the recipient embryo or by
30 exposure to irradiation. When the recipient embryos are compromised by a combination of irradiation and mechanical removal of the cells, the contribution of the ES cells is increased further, even though the cES cells had been in culture for longer periods of time. Some of the resulting chimeric chicks are indistinguishable from pure Barred Rock chicks (See Figure 3). As the data in Table 5 show, chimerism rates as well as the extent of chimerism per embryo
35 increases after compromising the recipient embryo.

Table 5: Frequency of somatic chimerism after injection of cES cells into recipient embryos that were compromised by different methods.

Treatment to compromise the recipient embryo	# Cell lines	Time cells in culture	# Chimeras	# Embryos & chicks evaluated	Frequency of chimerism %	Extent of feather chimerism (%)
None	14	4-106 days	83	347	24	26
Mechanical removal of cells	1	6 months	34	63	54	20
Irradiation	1	6-7 months	56	95	59	29
Irradiation & Mechanical removal of cells	1	7-8 months	52	59	88	49

Recipient embryos substantially younger than stage X may also be used to produce chimeras using ES cell as the donor. Early stage recipient embryos are retrieved by injecting the hens with oxytocin to induce premature oviposition and fertile eggs are retrieved at stages VII to IX.

Alternatively, the retrieval of embryos from the magnum region of the oviduct provides access to stage I to VI embryos, consisting of approximately 4-250 cells, and enables the development of chimeras from all embryonic stages as potential recipient embryos.

Example 3 – Somatic chimeras from chicken embryonic stem cells.

To demonstrate pluripotency, cES cells are injected into White Leghorn recipient embryos. In the first round, a total of 14 cell lines in 28 experiments are injected into stage X recipient embryos (See Table 2). The cES cells have been propagated in culture between 4 and 106 days and some lines had been cryopreserved. Chicken ES cells are lightly trypsinized, resulting in small clumps of cES cells, and resuspended in DMEM supplemented with 25mM HEPES + 10% fetal calf serum. Three to 5 μ l of the cell suspension, containing between 2000-5000 cells, are injected into the subgerminal cavity of the recipient embryos. All embryos that developed feathers are analyzed and twenty four percent of embryos (83/347) are chimeric as determined by feather color. Feather chimeras are obtained from 11/14 cell lines. The extent of chimerism varied from 1%-95% with a mean extent of 25.9% (SD=20.4).

Table 2 illustrates the variance in the somatic chimerism between experiments performed within and between cell lines. Examples of the contribution to chimeras is shown in Figures 3 and 4. In Figure 3, two chicks are chimeras and two are Barred Rocks; no phenotypic differences exist to indicate that the contribution of cES cells to the chimera is

extensive, particularly in the ectodermally derived lineages. In Figure 5, the chimeras on the left have relatively low levels of contribution from ES cells whereas those on the right have intermediate contributions.

5 Example 4 - Transfection of cES cells by lipofection or electroporation

Referring to Table 6, an appropriate amount of DNA compatible with the size of the well being transfected is diluted in media absent of serum or antibiotics. The appropriate volume of Superfect (Stratagene) is added and mixed with the DNA, and the reaction is allowed to occur for 5-10 minutes. The media is removed and the wells to be transfected are washed with a Ca/Mg free salt solution. The appropriate volume of media, which can contain serum and antibiotics, is added to the DNA/superfect mixture. The plates are incubated for 2-3 hours at 37C. When the incubation is completed, the Superfect is removed by washing the cells 1-2x and fresh culture media is added.

15 Table 6: Conditions for transfection of chicken ES cells using Superfect.

Plate Size	Volume of media used to dilute DNA	Total amount of DNA	ul Superfect	Time to form complex (min)	Volume of media added to complex	Incubation time
96 well	30 ul	1	5 ul	5-10	150	2-3 hrs
48 well	50 ul	1.5	9 ul	5-10	250 ul	2-3 hrs
24 well	60 ul	2	10 ul	5-10	350 ul	2-3 hrs
12 well	75 ul	3	15 ul	5-10	400 ul	2-3 hrs
6 well	100 ul	4	20 ul	5-10	600 ul	2-3 hrs
60 mm	150 ul	10	50 ul	5-10	1000 ul	2-3 hrs
100 mm	300 ul	20	120 ul	5-10	3000 ul	2-3 hrs

A petri-pulser is used to electroporate cES cells that are attached to the plate in a 35 mm diameter well. The media is removed and the well is washed with a salt solution without Ca^{++} and Mg^{++} . One ml of electroporation solution is added to the well. DNA is added and the media is gently mixed. The petri-pulser is lowered onto the bottom of the well and an electrical current is delivered. (Voltage preferably varies from 100-500 V/cm and the pulse length can be from 12-16 msec). The petri-pulser is removed and the electroporated well is allowed to stand for 10 minutes at room temperature. After 10 minutes, 2 mls of media is added and the dish is returned to the incubator.

25 To transfect cells in suspension, media is removed and cells are washed with a Ca/Mg free salt solution. Trypsin with EDTA is added to obtain a single cell suspension. Cells are washed, centrifuged and resuspended in a correctional electroporation buffer solution such as

PBS. The ES cell suspension is placed into a sterile cuvette, and DNA added (minimum concentration of 1mg/ml) to the cell suspension and mixed by pipetting up and down. The cells are electroporated and allowed to sit at RT for 10 minutes. Cells are removed from cuvette and distributed to previously prepared wells/dishes. Cells are placed in an incubator and evaluated or transient transfection 24-48 hours after electroporation. Selection of antibiotic resistant cells may also be started by including an antibiotic such as puromycin in the culture medium.

In a preferred embodiment, the concentration of puromycin required for selecting transfected cells is calculated as a titration kill curve. Titration kill curves for cES cells are established by exposing cells in culture to puromycin concentrations varying from 0.0 to 1.0 $\mu\text{g/ml}$ for 10 days (Table 7) and neomycin concentrations varying from 0.0 to 200 $\mu\text{g/ml}$ (Table 8). The medium is changed every 2 days and fresh puromycin or neomycin is added. When exposed to a concentration of 0.3 $\mu\text{g/ml}$ puromycin, cES cells were absent from all wells after 3 changes of medium with fresh puromycin over a six day period (see Table 7). Puromycin concentrations of 0.3-1.0 $\mu\text{g/ml}$ are used for selection of the transfected cultures. Neomycin concentrations over 40 $\mu\text{g/ml}$ eliminated all cES cells within 7 days (Table 8). After 10 days of selection, cES cells colonies are visible and can be picked for further expansion.

Table 7: Morphology of cES cells after exposure of various concentrations of puromycin and different lengths of time (days after addition of puromycin).

Puromycin conc. ($\mu\text{g/ml}$)	Time under selection (days)									
	1	2	3	4	5	6	7	8	9	10
0.0	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
0.1	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
0.2	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
0.25	ES	ES	ES	ES	ES	diff	diff	diff/	diff/	diff/
								gone	gone	gone
0.3	ES	ES	diff	diff/	diff/	gone	gone	gone	gone	gone
				gone	gone					
0.4	ES	diff	gone	gone	gone	gone	gone	gone	gone	gone
0.5	diff	gone	gone	gone	gone	gone	gone	gone	gone	gone
0.6	diff	gone	gone	gone	gone	gone	gone	gone	gone	gone
0.7	diff	gone	gone	gone	gone	gone	gone	gone	gone	gone
0.8	gone	gone	gone	gone	gone	gone	gone	gone	gone	gone

ES: ES cells are present. diff: ES cells are differentiated. gone: no morphologically recognizable cells are present

Table 8: Morphology of cES cells after exposure of various concentrations of neomycin and different lengths of time (days after addition of neomycin).

Neomycin conc. (μ g/ml)	Time under selection (days)									
	1	2	3	4	5	6	7	8	9	10
0.0	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
10	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
20	ES	ES	ES	ES	ES	ES	ES	ES	ES	ES
30	ES	ES	ES	ES	ES	ES	ES/ diff	ES/ diff	Diff	Diff/ gone
40	ES	ES	ES	ES	ES/ diff	Diff/ dead	dead	gone	gone	gone
50	ES	ES	ES	ES/ diff	ES/ diff	Diff/ dead	Dead/ gone	gone	gone	gone
60	ES	ES	ES	gone	gone	gone	gone	gone	gone	gone
100	ES/ diff	diff	dead	gone	gone	gone	gone	gone	gone	gone
150		dead	dead	gone	gone	gone	gone	gone	gone	gone
200		dead	gone	gone	gone	gone	gone	gone	gone	gone

Selection of transfected cES cells and their identification in chimeras requires that the transgene confer a selective advantage to the cells in culture (e.g. resistance to puromycin in the medium) and produce an identifiable gene product in the cells in the chimera which are derived from the ES cells. This can be accomplished using pCX/GFP/Puro which provides resistance to puromycin in cES cells and produces a green fluorescent protein (GFP) in most, if not all, donor-derived cells in chimeras.

Referring to Figure 5, PCX/GFP/Puro was produced in three cloning steps involving two intermediates before make the final pCX/GFP/Puro plasmid. In step 1, the PGK-driven Puromycin resistant gene cassette (1.5 Kb) was released from pKO SelectPuro (Stratagene) by Asc I digestion. The fragment was then blunted and Kpn I linkers were added. The resulting fragment (GFP/Puro) was inserted into the corresponding Kpn I site of pMIEM (courtesy of Jim Petitte (NCSU), a GFP expression version derived from LacZ expression pMIWZ, see Suemori et al. *Cell Diff and Dev.* 29: 181-186 (1990) to produce the first intermediate (pGFP/Puro). The PGK-Puro cassette was in same transcription orientation as GFP (determined by BamH I and Sty I digestion). In step 2, the GFP/Puro expression cassette (2.5 Kb) was released from pGFP/Puro by BamH I and EcoR I double digestion. The resulting fragment was inserted into the BamH I and EcoR I sites of pUC18 (Invitrogen). It contains 5' unique sites, Hind III, Pst I and Sal I. The resulting plasmid pUC18/GFP/Puro was verified by a BamH I, EcoR I, and Not I triple digestion. In the third step, the Cx promoter including 384 bp CMV-IE enhancer, 1.3 kb chicken beta-actin promoter and portion of 1st intron was released from pCX-EGFP (Masahito, I. et al., *FEBS Letters* 375: 125-128, 1995) by Sal I and

EcoR I digestion. A 3' EcoR I (null)-Xmn I-BamH I linker was attached to the fragment and it was inserted into the Sal I and BamH I sites of pUC18/GFP/Puro. The plasmid pCX/GFP/Puro was verified by a BamH I and Pst I double digestion. pCX/GFP/Puro DNA can be linearized by Sca I digestion for transfection into cES cells.

5 Transfection and selection of ES cells using the procedures described above produced a population of cells that would grow in the presence of 0.5 ug of puromycin. These cells exhibited green fluorescence when examined by conventional fluorescence microscopy. Referring to Figure 6, when preparations of the cES cells are examined by FACS, essentially all of the cells carry and express the transgene. Southern analysis of DNA from the transfected
10 ES cell lines TB01 and TB09 that was digested with BamH1, EcoRI or both restriction endonucleases revealed the transgene in DNA fragments of various sizes, providing evidence that the transgene is integrated into the genome (See Figure 7).

The CX/GFP/Puro construct demonstrates that transgenes of at least 4.5kb can be inserted into chimeric chickens. Using the cES cells described herein, chicken ES cells can be
15 transfected with different or larger constructs. In the preferred embodiments of the invention, the transgene is comprised of DNA encoding an exogenous protein that is present and widely distributed in somatic cells of the resulting chimeric or transgenic animal. Preferably, the transgene is present and expressed in any or all of the endoderm, mesoderm, ectoderm, and extra embryonic tissue. The DNA encoding the exogenous protein is preferably expressed in
20 the tissue types such that the exogenous protein is detectable in the specific cell types.

In another example, ovalbumin constructs designed to express significant levels of rearranged immunoglobulin molecules in the tubular gland cells of the magnum region of the oviduct are inserted into cES cells and used to make chimeras. Ovalbumin-derived monoclonal antibody expression constructs containing 42 to 49 kb of DNA are designed from a chicken
25 genomic BAC library (Crooijmans, R. P. Mamm. Genome 11: 360 (2000), (Texas A & M BAC Center) and are screened to isolate a 40 Kb region in the ovalbumin locus.

Two ovalbumin-derived expression vectors are constructed based on above identified sequences: (1) Ov7.5MAbdns: A 42 Kb expression vector contains 9.2 Kb 5' sequences from the ovalbumin gene (including 7.5 Kb promoter) and 15.5 Kb 3' flanking sequences (Figure
30 8A). This 42 kb expression vector contains 9.2 kb of 5' sequence from the ovalbumin gene (including 7.5 kb promoter) and 15.5 kb 3' flanking sequences. A bicistronic monoclonal antibody cassette encodes the light chain, an IRES and the heavy chain of an anti-dansyl antibody.

(2) Ov15MAbdns: A 49 Kb expression vector contains 16.8 Kb 5' sequences from the
35 ovalbumin gene (including 15 Kb promoter) and 15.5 Kb 3' flanking sequences (Figure 8B).

The 49 kb expression vector contains 16.8 kb of 5' sequence from the ovalalbumin gene (including 15 kb promoter) and 15.5 kb 3' flanking sequences. The monoclonal antibody cassette is identical to the above. The expressed gene in both vectors is a mouse-human hybrid anti-dansyl monoclonal antibody (MAbdns). A CxEGFP/CxPuro cassette is cloned in the most 3' end to allow selection with puromycin for stable transfection in cES cells and easy identification of transfected cells in chimeras. Both constructs are linearized and purified before transfection.

Transfection is performed with Ov7.5MAbdns and Ov15MAb using either SuperFect (Stratagene) or petri-pulser electroporation. After selection with puromycin, 6 resistant clones are picked for molecular analysis. The presence of the transgene is evident by PCR with primers located in the MAbdns cassette, in the GFP gene, and in the Puro gene.

In yet another example, a very large transgene encoding part of the unrearranged human heavy chain immunoglobulin locus has been transfected into chicken ES cells. A 139 kilobase bacterial artificial chromosome (BAC) clone was co-transfected with the pCX-EGFP-CX-puro selectable marker into cES cells by co-lipofection of circular BAC DNA and linear selectable marker DNA. The BAC clone contains a human genomic DNA insert from an unrearranged immunoglobulin heavy chain locus and contains the most 3' variable region (V_H6-1), all the diversity (D) segments, all the joining (J) segments, the C_μ and C_γ constant regions, the J-intronic enhancer, and all the intervening DNA between these elements. It also includes the human gene KIAA0125, a gene that encodes a non-translated RNA of unknown function that is found between V_H6-1 and the D segment region. pCX-EGFP-CX-puro is a plasmid that contains the Enhanced Green Fluorescent Protein (EGFP) gene driven by the CX promoter (consisting of a cytomegalovirus enhancer and the chicken β-actin promoter) and a puromycin resistance gene driven by the same promoter. The cES cells transfected with this plasmid are green fluorescent and resistant to the antibiotic puromycin. The presence of the unrearranged human heavy chain locus in the transfected ES cells that were growing in the presence of puromycin was examined by PCR analysis of transgenes spread throughout the 139 kb construct. The primer sequences used in the PCR analysis were:

V_H6-1:

V6-1F AGTGTCAAGGAGATGCCGTATTCA

V6-1R ACTCCCCCTCACTGTGTCTCTTG

D1-26:

D1-26F GGGCGCCTGGGTGGATTCTGA

D1-26R GTGGCCCCTAACCTGAGTCTGCT

D1-20:

D1-20F CCCGAGCACCGTCCCCATTGA

D1-20R GTGCCGGTGATCCCTGTCTTTCTG

C_μ:

Mu1F GCGGGAGTCGGCCACCATCAGC

Cδ:
Delta1F TGGGGAGAGGAGAGCACAGT
Delta1R GGCGGGCGTAGGGGTCAGC

cES cells are co-transfected with the selectable marker pCX-EGFP-CX-puro and the BAC CTD-2005N2, resulting in a cES cell line designated BAC-A. Genomic DNA is prepared and PCR performed using 5 different primer sets corresponding to markers along the length of the BAC clone. These markers are: V_H6-1 (24 kb from the 5' end -- relative to the human heavy chain locus -- of the human genomic insert), D1-26 (83 kb from the end), D1-20 (73 kb from the end), C_μ (~108 kb from the end), and Cδ (~120 kb from the end). Only V_H6-1, C_μ, and D1-26 are shown but all gave similar results. As a control for amplification from the cES cell sample, a chicken β-actin PCR is also run. The samples are: (1) BAC-A cells; (2) mouse STO cells used as a feeder layer for the cES cells (negative control); (3) barred rock embryo DNA (the same strain as the parental cES cell line, negative control); (4) human genomic DNA (positive control); and (5) cES cell medium (negative control).

As shown in Figure 9, all segments of the transgene are present in the transfected and selected ES cells.

Example 5 - Identification of transfected donor-derived cells in chimeras

Transfected donor derived cells are detected in chimeras by a number of methods. For example, the transgene is detected by Southern analysis of tissue taken from chimeras that are identified by the presence of black pigmentation in their feathers. Also, DNA is harvested from embryos or from comb tissue and digested with BamHI, EcoRI or a combination of these endonucleases. When DNA from tissues in the chimeras are examined by Southern analysis, bands that were identical to those seen in TB01, which was the donor cell used to make the chimeras, were evident after digestion with either BamHI, EcoRI or a combination of these two restriction endonucleases (Figure 10), thus providing evidence that the chimera contains progeny of the ES cells that were introduced into the recipient embryo to form the chimera.

The presence of the transgene in tissues is detected by illuminating the chimeras with fluorescent lights, which shows that the transgene is expressed in the eye and the beak, the buccal cavity, legs and feet, and in the bones of the wing and in cells in the feather pulp. Examination of the internal organs revealed that donor-derived ES cells had contributed to the intestinal tissues and breast muscle and leg muscle. In another bird, extensive contributions to the pancreas were observed. These data provide compelling evidence that the progeny of ES

cells contribute to the ectoderm which gives rise to the beak, feathers and skin, to the mesoderm which gives rise to muscle and bone and to the endoderm, which gives rise to the pancreas.

Illumination of the bird with fluorescent light is used to score the birds on a scale of 1 to 4 where 4 indicates that all of the visible skin, eyes and comb are fluorescent. The fluorescent score was determined by screening several areas of the skin with a fluorescent lamp and scoring the chicks on a scale of 0 to 4. A similar score is used to rate the extent of chimerism estimated by feather pigmentation. Feather chimerism was estimated as % of black down compared to down at hatch. Approximately 25% of the down of a Barred Rock chick is white. The maximum score that is possible in a chimera derived completely from Barred Rock derived ES cells is, therefore, 75%. The correlation between the two which is described by the equation $y = 0.41x + 0.25$ where y is the fluorescence score and x is the extent of ES-cell derived feather pigmentation is shown in Figure 11.

Examination of the chorioallantois indicated that cES cells also contributed to the extraembryonic tissues. These data indicate that the pluripotentiality of cES cells may be greater than murine ES cells, which do not contribute to the trophectoderm.

In addition to examination of live tissues in chimeras, cells were prepared from a variety of tissues and the proportion of cES-cell derived fluorescing cells was determined. Tissue (muscle, liver or brain) was removed from chickens post-mortem, then rinsed with PBS to remove blood or other fluids. The outer membrane of the tissue was dissected away and the remaining sample was minced. Each tissue sample was transferred to a microcentrifuge tube containing 1 ml of 1 mg/ml collagenase (Type IV, Sigma) in either DMEM (muscle and liver) or Liebovitz L15 medium (brain) without serum. These tubes were incubated in a 37°C water bath for 30-45 minutes (the tubes were inverted every 10-15 minutes to shake the suspension) and then the tissues were dispersed into single cell suspensions using a 100-200 ul pipette equipped with a disposable plastic tip and purified according to the tissue type. All centrifugation steps were executed in a microcentrifuge.

For the muscle cell suspension, the suspension was centrifuged for 5 min at 4000 rpm, the supernatant removed and replaced with 500 ul of DMEM containing 10% heat inactivated horse serum. The pellet was resuspended, and the tube centrifuged for a further 60 seconds at 2600 rpm, the supernatant was then removed and filtered through 40 micron nylon mesh (sterile disposable, Falcon brand). A sample of the cell suspension was inspected by microscopy at this stage to ensure correct cell morphology and sufficient cell density for flow cytometry (sub optimal densities were corrected by dilution, or by pelleting and resuspension).

For the liver cell suspension, the suspension was filtered through 40-micron nylon mesh, transferred to a clean microcentrifuge tube and pelleted as for muscle. The top layer of the pellet was carefully removed to a clean tube with complete DMEM (500 ul), taking care not to disturb the red blood cells which form a distinct and visible layer at the bottom of the pellet. The cell suspension was inspected at this stage and the cell density adjusted. If red blood cells were present in the cell preparation they were removed with a lysis step (incubating the cells in 1 ml lysis buffer containing 130 mM Ammonium Chloride, 17 mM Tris, 10 mM Sodium Bicarbonate for 5 minutes at room temperature).

For the brain cell suspension, the suspension was filtered and pelleted as described above. The pellet was resuspended in 250ul complete Liebowitz (L-15) medium containing 10% horse serum and 6g/l glucose, Percoll was added to produce a 50% solution (approx. 260 – 280 ul). The suspension was centrifuged at 3.5k for 5 minutes, following which the top layer of cells was carefully removed into a clean microcentrifuge tube and diluted with at least 1 ml of L-15 medium. The cells were pelleted by centrifuging at 4000 rpm for 5 minutes and then were resuspended in an appropriate volume of L-15.

A flow cytometric analysis of GFP expression was conducted in liver, brain and muscle cells from chimeras. Single cell suspensions were transferred into polystyrene tubes and loaded into a flow cytometer, for which the operating parameters had been set to detect the particular cell type of the sample, and which was equipped to detect emitted light at a green wavelength in response to an excitory laser beam. At each analysis, at least one group of samples from a non-transgenic chimera was analyzed in order to set the baseline for fluorescence measurement (since the flow cytometer detects some autofluorescence from each cell). These are referred to as control samples in Figure 12. The data produced by the flow cytometer included the number of cells detected (within the parameters specified) and summarized the fluorescence of the cells in that population. Examples of the data from analyses of brain and muscle tissue is shown in Figures 13 and 14, respectively. Data was collected for the number of cells which exhibited a fluorescence intensity greater than the autofluorescent level set by the non-transgenic sample for each tissue type (designated M1). A subset of these data was collected for cells which exhibited a fluorescence intensity at least ten times greater than the autofluorescent level (designated M2).

Brain, liver and muscle (breast and leg) samples were removed from twenty six chickens, of which 18 were chimeras that had been produced by injecting transgenic chicken ES cells carrying the GFP transgene into non-transgenic White Leghorn recipient embryos, as described above. The remaining 8 chickens were chimeras that were produced by injecting non-transfected cES cells into non-transgenic White Leghorn recipient embryos. Male and

female chickens were present in both the groups. Green fluorescence was detected in brain-derived, liver-derived and muscle-derived cells from the transgenic chimeras. The fluorescence intensity and the ratio of fluorescent to non-fluorescent cells varied between birds and between tissue type. The tabulated results are shown in Figure 12. In birds which had been
5 awarded a low score for donor-derived feather pigmentation, or for green skin when screened with a UV lamp prior to post-mortem analysis, the number of fluorescent cells in the tissue samples was generally low or zero. Birds which scored well for these criteria (e.g. approximately 75% donor-derived feathers) were found to have fluorescent cells in all three tissue sample types, and this was the only group in which highly fluorescent (M2) cells were
10 present in all three tissue types. Of the three tissue types, the number of fluorescent cells derived from the liver was the lowest. Brain and muscle-derived fluorescent cells were present in greater numbers and in a greater number of samples derived from the transgenic chimeras. The data is shown graphically in Figure 11.

The data shows that transgenic cES cells, when injected into recipient stage X embryos,
15 can thrive in the host and differentiate into liver, muscle and brain cells. Liver, muscle and brain are tissues that are derived from endoderm, mesoderm and ectoderm, respectively; thus cES cells may differentiate into the three major somatic cell lineages, from which all other somatic cells are derived. Further, this experiment shows that transgenic cES-derived cells persist beyond the embryonic stages and can be seen in juvenile chickens, and that the
20 transgene continues to express in these diverse cell types.

The transgene is also present in lymphocytes derived from cES cells. The hemopoietic lineage, which comprises the lymphoid and myeloid lineages, is of particular interest in transgenic chimeras derived from ES cells because B cells in the lymphoid lineage produce antibodies. Lymphocytes are prepared either from blood samples taken from chimeric
25 chickens at any time from hatching to adulthood or from the Bursa of Fabricius of chimeric embryos. Bursae are removed from chicks at day 20 of embryonic development (E20) and macerated by forcing through steel mesh in 10 ml of Hanks' Buffered Salt Solution (HBSS) with the plunger of a 20 ml syringe. The resulting tissue fragments and cells are collected into a tube and incubated at room temperature for 5 minutes to allow the large fragments to settle.
30 The cell supernatant is harvested and the cells are counted and then collected by centrifugation at 1500g for 10 minutes at 4°C and resuspended at a maximum of 1×10^8 cells per ml of HBSS in a 15 ml conical tube. Blood is collected (0.5 ml) from chimeric chickens' wing veins using a heparinized syringe and deposited into a vacuum tube containing EDTA to prevent clotting. Blood samples are mixed 1:1 with HBSS to give a final volume of 1 ml in a 15 ml conical tube.
35 From this point on, blood samples and Bursa samples are treated the same way. One ml of cell

suspension is underlayered with 0.75 ml Fico/Lite-LM (Atlanta Biologicals catalog number I406) by dispensing the Fico/Lite at the bottom of the tube, underneath the cell suspension. The tubes are then centrifuged at 1500g for 15 minutes at 4°C, no brake. The interface between the Fico/Lite and the HBSS is carefully harvested to collect the mononuclear cells that have concentrated there in a discrete layer of material. This material is transferred to a new tube, triturated to break up the compacted cells, and then mixed with 3 ml of HBSS/2% heat inactivated fetal bovine serum. The cells are collected by centrifugation at 1500 rpm in a Sorvall benchtop centrifuge, 10 minutes at 4°C, then washed two more times in HBSS/2% FBS. A small aliquot (25 µl) is mounted on a microscope slide for preliminary assessment of the extent of donor-derived GFP fluorescence under the microscope. The remainder of the cells are then ready for antibody staining or fixation.

To store cells for longer than a few hours before analysis or if they are to be permanently mounted on slides for microscopy, they are first fixed in paraformaldehyde. An aliquot of cells (50 µl, $0.5-1 \times 10^6$ cells) is fixed by adding 1 ml 4% paraformaldehyde and incubating at room temperature for 15 minutes. The cells are then washed three times by centrifugation at 500g for 6 minutes in a microfuge to collect the cells, followed by resuspending the cells in PBS/2% heat inactivated fetal bovine serum.

In antibody staining, an aliquot of 0.5×10^6 cells in fresh PBS/2% FBS/0.1% sodium azide is placed in a tube or a well of a 96-well plate, on ice. Monoclonal antibodies conjugated to R-phycoerythrin (Southern Biotechnology Associates) are added to the cells, incubated for 30 minutes on ice, and covered to protect the fluorophore. Antibodies that recognize the chicken B lymphocyte marker Bu-1 (used at a working dilution of $0.2\text{g}/10^6$ cells) or the chicken T cell marker CD3 (used at $0.5\text{g}/10^6$ cells) are used. After incubation the cells are washed three times by centrifugation at 500g for 6 minutes, resuspending in 0.5 ml PBS/2% FBS/0.1% azide each time. After the final wash the cells are stored in 0.5% paraformaldehyde up to one week before analysis by flow cytometry. The paraformaldehyde is replaced with PBS/2% FBS on the day the cells are to be analyzed by flow cytometry; buffers without phenol red are used for flow cytometry. FACS analysis is performed for both GFP fluorescence and R-phycoerythrin fluorescence simultaneously, to detect the total proportion of cells that are donor derived (GFP-positive), the proportion of cells (both donor and recipient derived) that are stained by the antibodies (R-phycoerythrin-positive), and the proportion of donor derived cells that are also stained with the antibodies (GFP, R-phycoerythrin double positive).

Chimeras made with three different ES cell lines were analyzed for donor contribution to the lymphoid lineage, representing three different insertion sites for the CX-GFP marker

(Table 9). A total of 27 chimeras have been analyzed, ranging in age from pre-hatch to adult. The proportion of donor-derived GFP-positive cells in the lymphocyte fraction ranged from 0 to 10% as judged by FACS analysis (animals with 0% are not shown in the table). Antibody staining of peripheral blood lymphocytes resulted in 5-17% of the cells staining with anti-Bu-1 antibodies and 75-85% of the cells staining with anti-CD3 antibodies. Staining of bursal lymphocytes with anti-Bu-1 antibodies resulted in over 90% of the cells stained. Double positive cells with GFP fluorescence and staining for either Bu-1 or CD3 and GFP were observed at low frequencies in several of the samples.

These data show that ES cells containing a gene encoding an exogenous protein contributed to the hemopoietic lineage in hatched chicks and mature animals.

Table 9. Contribution of cES cells to the lymphocyte lineage in somatic chimeras.

<u>Chimera</u>	<u>Age</u>	<u>GFP</u>	<u>Bu-1+</u>	<u>CD3+</u>	<u>GFP+</u> <u>and Bu-</u> <u>1+</u>	<u>GFP+</u> <u>and</u> <u>CD3+</u>	<u>Sex</u>	<u>Feather</u>	<u>Green</u> <u>score</u>	<u>Cell</u> <u>line</u>
OV-36	10	10	ND	ND	ND	ND	F	15	ND	OVF
OV-21	10	7	5	85	0	0	M	0	ND	OVF
10881	67	5	17	75	0.03	1	M	60	3	TB01
10891	49	3	12	74	0	0.1	F	5	1	TB01
10821	111	1	ND	ND	ND	ND	M	75	3	TB01
10877	98	1	11	ND	0.04	ND	F	75	4	TB01
IG1-25	E20	0.2	95	ND	0.03	ND	F	70	ND	BAC A
10845	146	0.18	8.5	ND	0	ND	M	60	3	TB01

The eight chimeras in Table 8 contain cES cell-derived lymphocytes. Peripheral blood lymphocytes (or bursal lymphocytes in the case of chimera IG1-25) were prepared with Fico/Lite-LM as described above. Lymphocytes were then analyzed for expression of green fluorescent protein (GFP), the Bu-1 B cell associated alloantigen (Bu-1), and the CD3 member of the T cell receptor-associated CD3 complex (CD3). The numbers in the columns labeled with GFP, Bu-1 and CD3 indicate the percentage of cells in each sample that were positive for those markers by FACS analysis (except for OV-36 and 10821 in which the percentage of GFP fluorescing cells was determined by counting cells under the microscope). The age of the chimeras when sampled is indicated in days, and the sex is indicated. The column labeled "Feather" indicates the percentage estimate of black, cES cell derived feather pigmentation, with 75 being the highest amount of black possible (as pure Barred Rock chickens themselves have a plumage that is approximately 75% black). "Green score" is a subjective evaluation of overall green fluorescence in the whole animal visualized by shining a handheld UV lamp on

the live animal with a scale from 0 (no green) to 4 (the most green). This score is used as a further indication of the overall extent of cES cell contribution in each animal. "Cell line" indicates the names of the different cES cell lines.

- 5 Various modifications, improvements, and applications of the disclosed invention are apparent to those of skill in the art are encompassed by the present application. The full scope of the invention is so limited by the preferred embodiments, but extends to the full scope of the claims.

WE CLAIM:

1. A composition comprising a sustained culture of chicken embryonic stem cells with a transgene stably integrated into the genome of substantially all of the progeny of the embryonic stem cells and wherein the embryonic stem cell progeny contribute to a somatic tissue of a chimeric chicken produced by the injection of the chicken embryonic cells into a chicken embryo.
2. The composition of claim 1 further comprising a feeder layer of cells at a concentration of between approximately 10^3 and 10^5 cells/cm².
3. The composition of claim 1 further comprising BRL conditioned media or fetal bovine serum.
4. The composition of claim 1 wherein pluripotency is sustained for at least 180 days.
5. The composition of claim 1 wherein the integrated transgene encodes an operably linked promoter, a selectable marker and an exogenous protein.
6. The composition of claim 5 wherein the integrated transgene is greater than 15 kb.
7. The composition of claim 1 wherein the somatic tissue is mesodermal.
8. The composition of claim 1 wherein the mesodermal tissue is lymphocytes or oviduct.
9. A chimeric chicken comprised of embryonic stem cell progeny having a stably integrated transgene.
10. The chimeric chicken of claim 9 wherein the transgene is further comprised of an operably-linked promoter and a selectable marker.
11. The chimeric chicken of claim 9 wherein the transgene is further comprised of DNA encoding an exogenous protein.
12. The chimeric chicken of claim 11 wherein the size of the transgene is greater than 15 kb.

13. The chimeric chicken of claim 11 wherein the exogenous protein is expressed in somatic tissue of the chicken.

14. A method of creating a chimeric chicken comprising:
selecting chicken embryonic stem cells from a culture sustained for more than 60 days wherein the embryonic stem cells have a genome comprising a stably integrated transgene,
injecting the stem cells into a recipient embryo, and
hatching a chimeric chicken from the recipient embryo wherein the transgene is present in somatic tissue of the chimera.

15. The method of claim 14 wherein the exogenous protein encoded by the transgene is expressed in the somatic tissue of the chimera.

16. The method of claim 14 wherein the transgene is comprised of a promoter and an operably-linked selectable marker.

17. The method of claim 16 wherein the transgene is further comprised of DNA expressing an exogenous protein.

18. A method to produce a chimeric chicken having expression of an exogenous protein in a somatic tissue comprising:
preparing a sustainable culture of chicken embryonic stem cells,
transfecting the embryonic stem cells with a transgene encoding an exogenous protein,
selecting embryonic stem cells in which the transgene is stably integrated into the genome,
injecting the embryonic stem cells into a recipient embryo;
hatching the chimeric chicken wherein the somatic tissue of the chimeric chicken expresses the exogenous protein.

Fig. 1

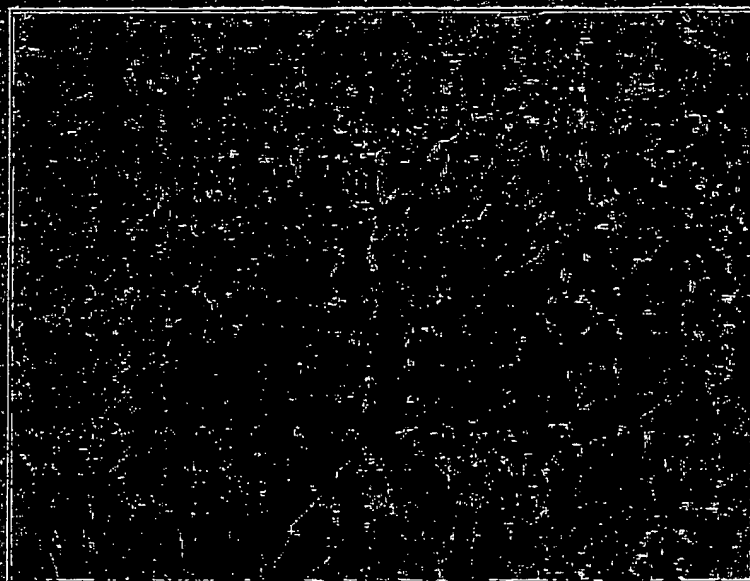


Fig. 2

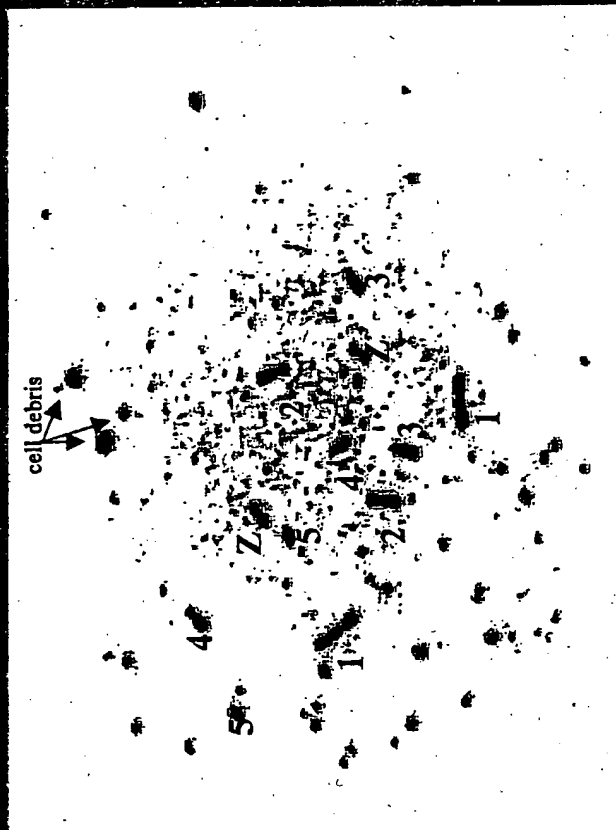


Fig. 3

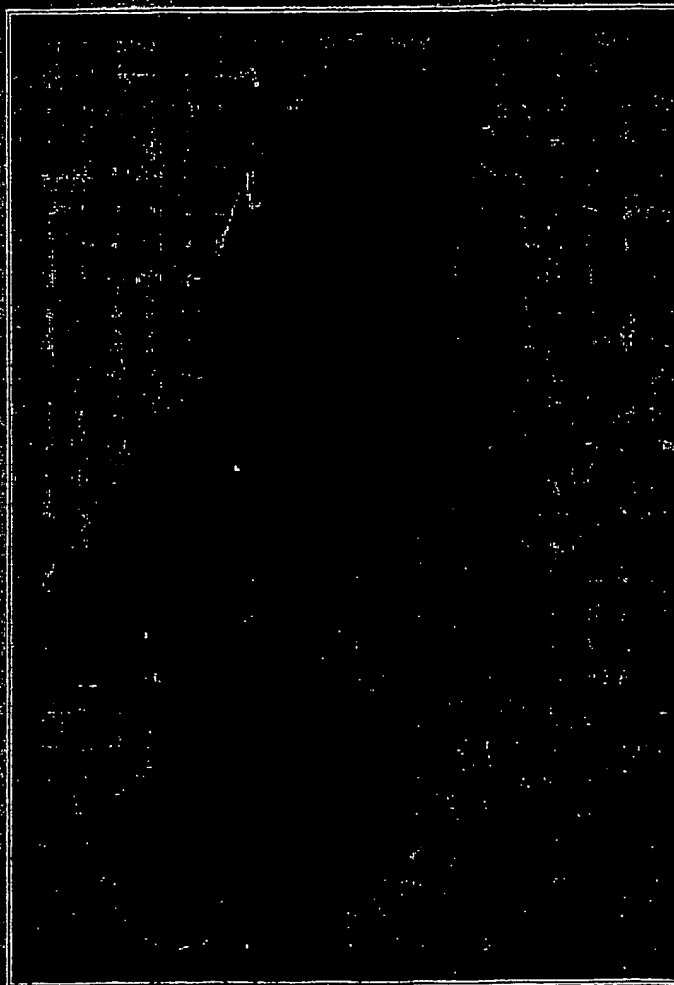


Fig. 4

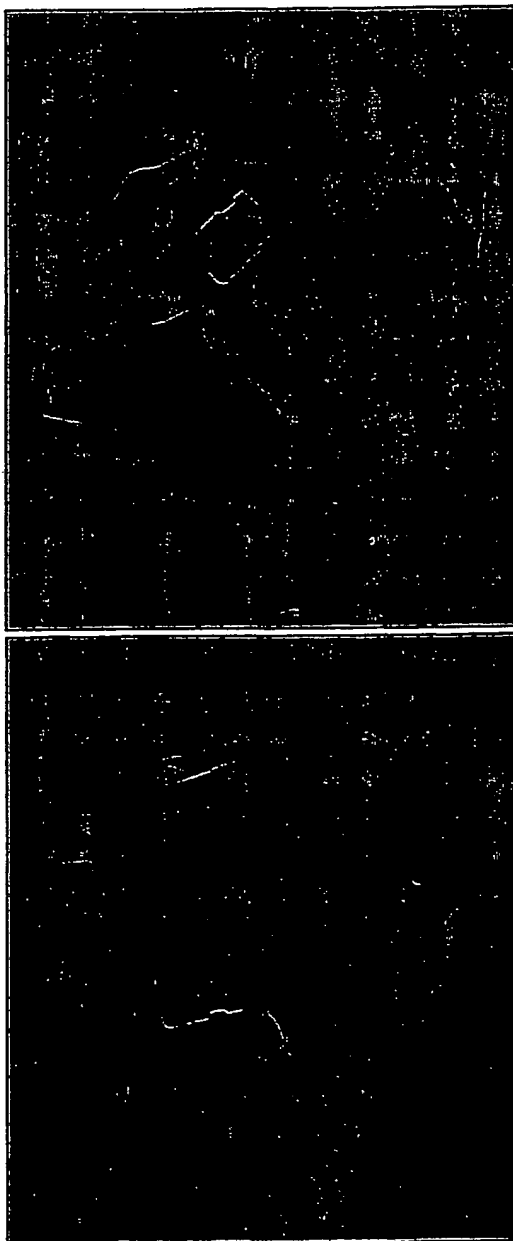


Fig. 5

pCxGFP/Puro

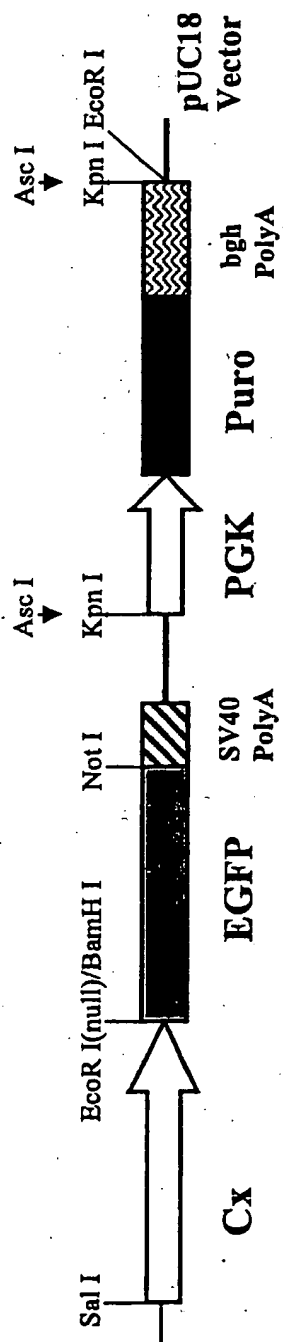
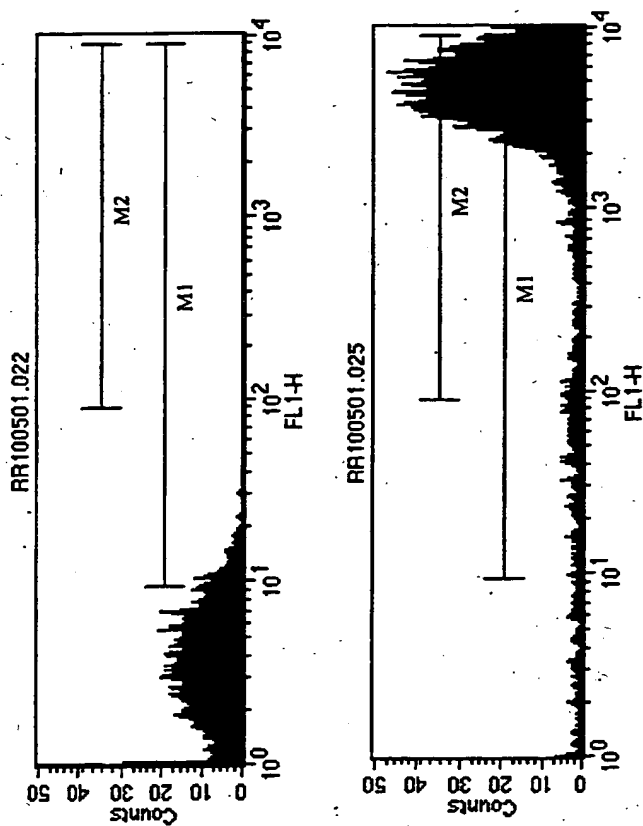
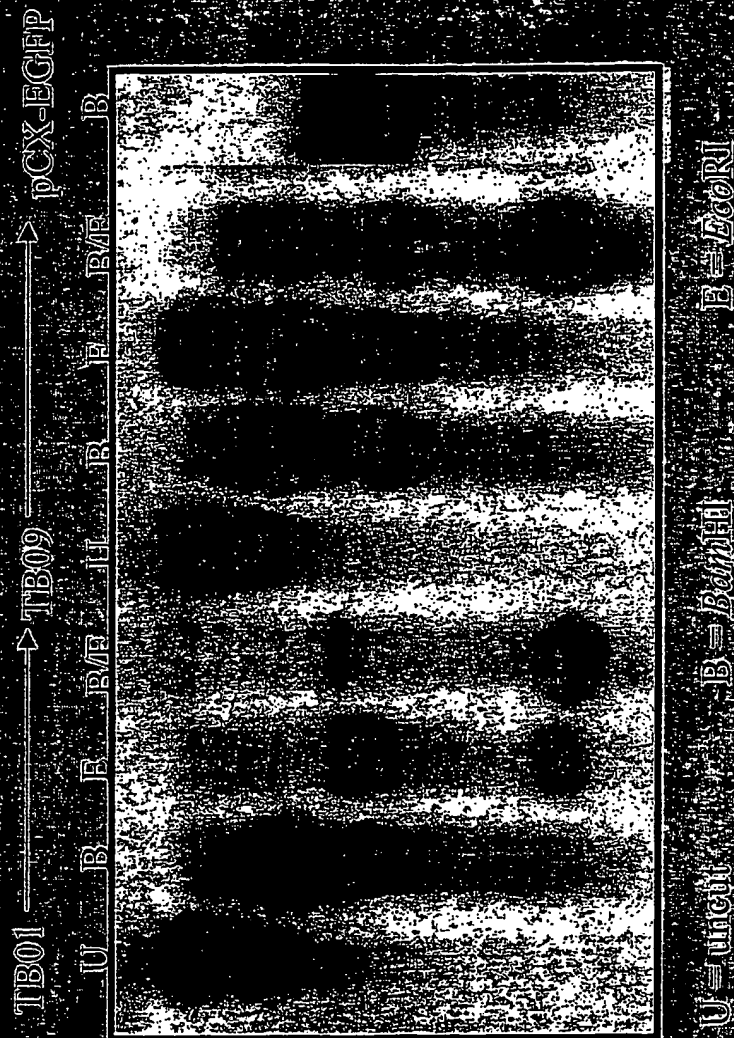


Fig. 6

1.

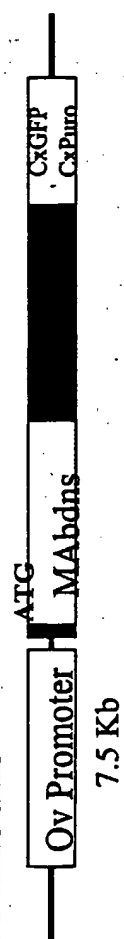
2.

Fig. 7

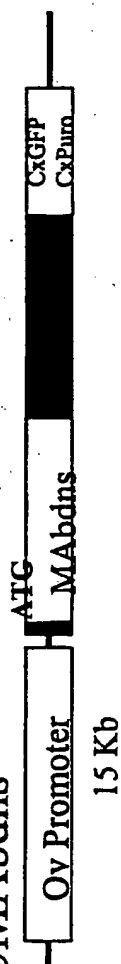


Figs. 8A and 8B

A. Ov7.5MAbdns



B. Ov15MAbdns



PCR analysis of BAC-A ES cells

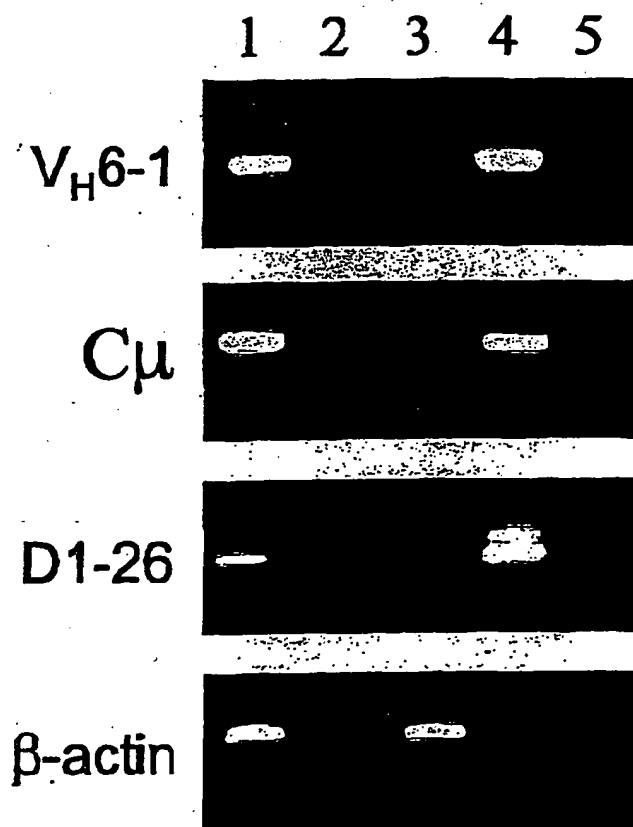
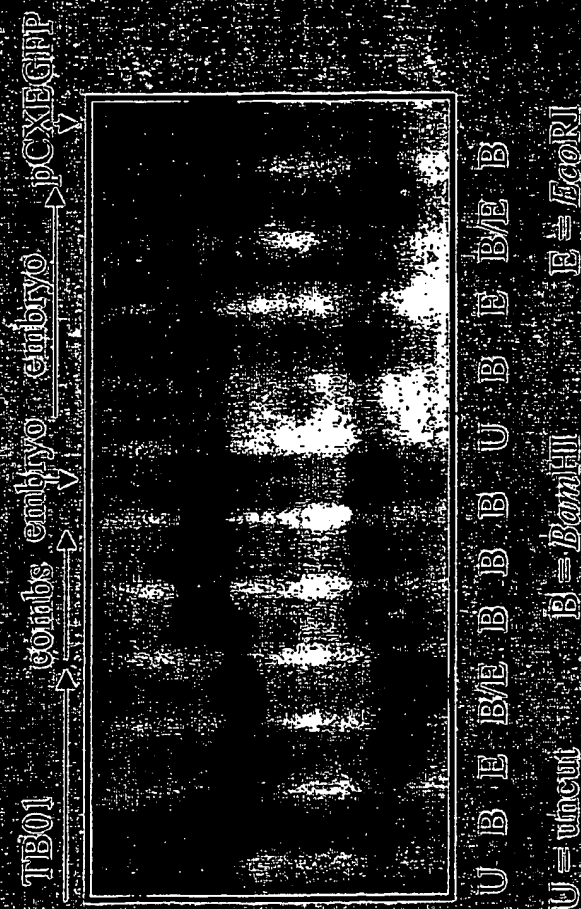


Fig. 9

Fig. 10.



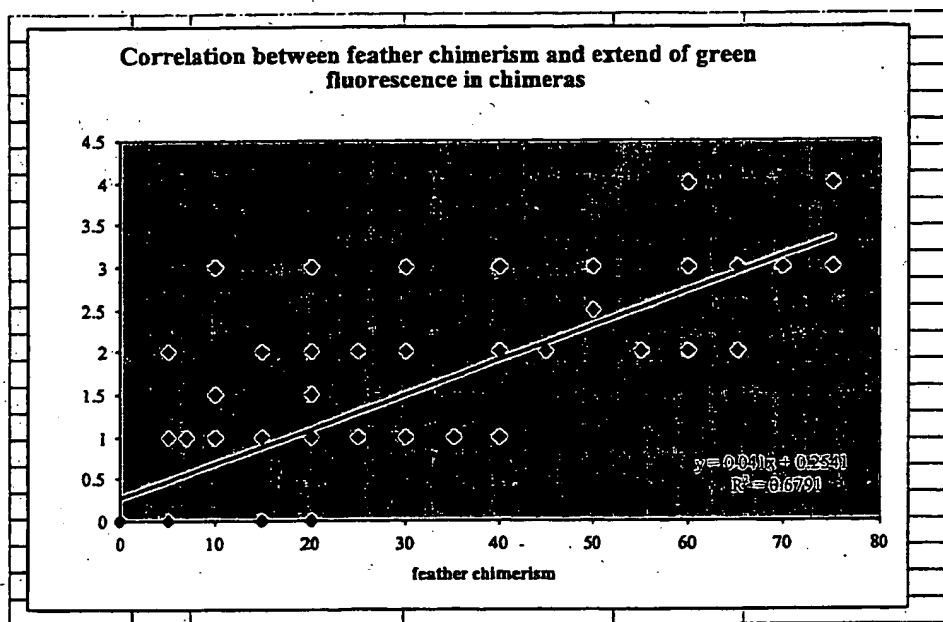


FIGURE 11

FIGURE 12

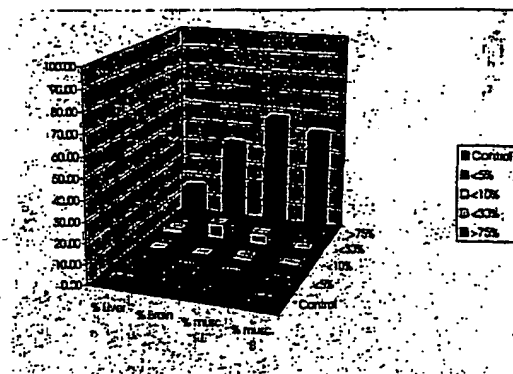
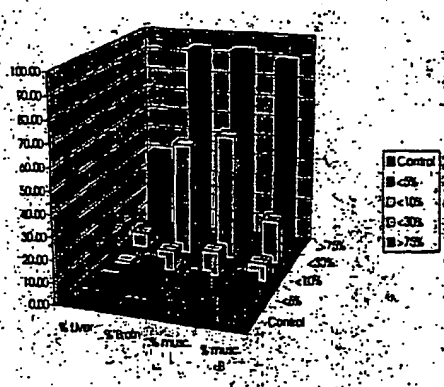
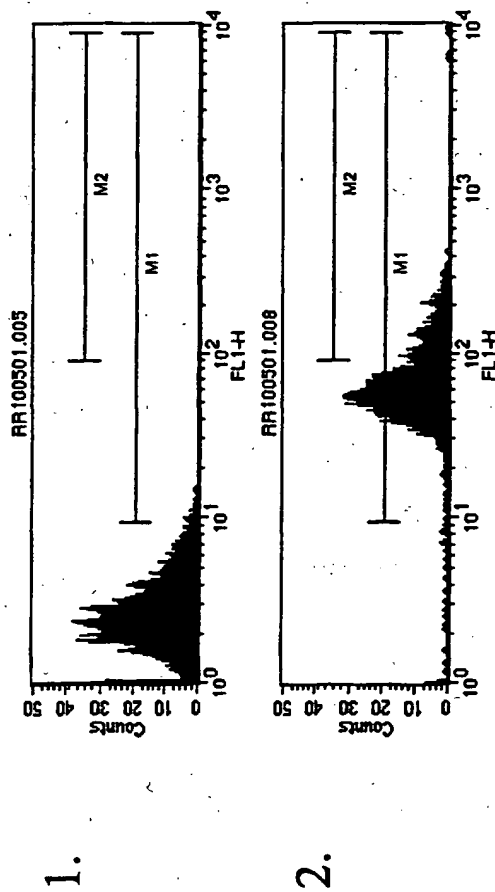


Fig. 13

Flow cytometric analysis of green fluorescence from Dispersed brain cells from juvenile chimeras. Cell count on y-axis, fluorescence intensity on x-axis.

- 1 - chimeras made with untransfected cES cells
- 2 - chimeras made with cES cells stably transfected with GFP

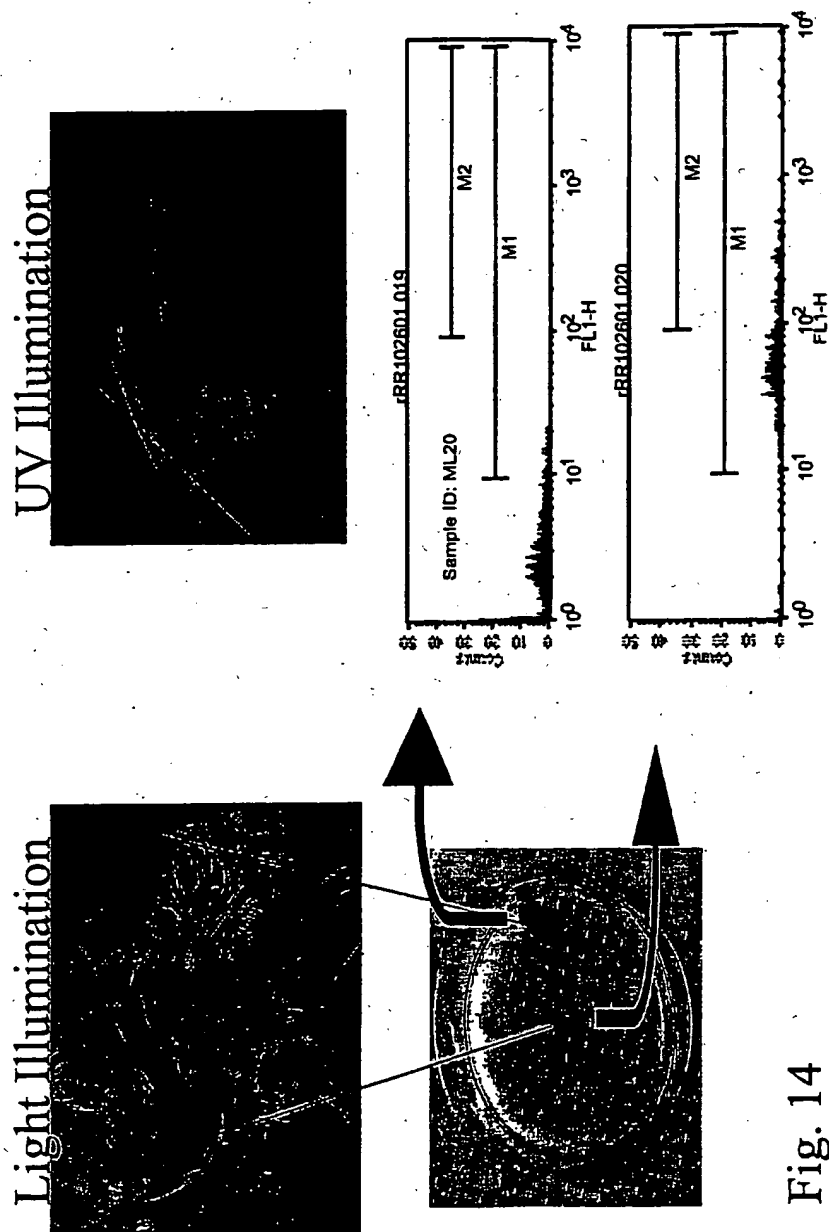


Fig. 14

SEQUENCE LISTING

<110> van de Lavoir, Marie-Cecile
Etches, Robert J.
Heyer, Babette
Mather, Christine
Diamond, Jennifer
Beemer, Kathleen
Meyers, Heather

<120> CHIMERIC BIRD FROM EMBRYONIC STEM CELLS

<130> 271/123 -- KTM

<140> US 10/067,148

<141> 2002-02-01

<160> 10

<170> PatentIn version 3.1

<210> 1
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> VH6-1 forward primer

<400> 1
agtgtcaggg agatgccgta ttca
24

<210> 2
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> VH6-1 reverse primer

<400> 2
acttcccctc actgtgtctc ttg
23

<210> 3

<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> D1-26 forward primer

<400> 3
gggcgcctgg gtggattctg a
21

<210> 4
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> D1-26 reverse primer

<400> 4
gtggccccta aacctgagtc tgct
24

<210> 5
<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> D1-20 forward primer

<400> 5
cccgagcacc gtccccattg a
21

<210> 6
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> D1-20 reverse primer

<400> 6
gtgccggtga tccctgtctt tctg

24

<210> 7
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> C-mu forward primer

<400> 7
gcgggagtcg gccaccatca cg
22

<210> 8
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> C-mu reverse primer

<400> 8
agcacagccg ccgccccagt ag
22

<210> 9
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> C-delta forward primer

<400> 9
tggggagagg agagcacagt
20

<210> 10
<211> 19
<212> DNA
<213> Artificial sequence

<220>

<223> C-delta reverse primer

<400> 10
ggcgggcgta ggggtcagc
19

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 August 2003 (07.08.2003)

PCT

(10) International Publication Number
WO 03/064627 A3

(51) International Patent Classification⁷: A61K 67/00,
67/027, C12N 15/00, 5/06, 15/63

(21) International Application Number: PCT/US03/03235

(22) International Filing Date: 3 February 2003 (03.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/067,148 1 February 2002 (01.02.2002) US

(71) Applicant: ORIGEN THERAPEUTICS [US/US]; 1450
Rollins Road, Burlingame, CA 94010-2307 (US).

(72) Inventors: ETCHES, Robert, J.; 111 East 9th Avenue,
Apt. 103, San Mateo, CA 94401 (US). VAN DE LAVOIR,
Marie-Cecile; 22 Gaviota Way, San Francisco, CA 94127
(US). HEYER, Babette; 806 Coleman Avenue, #5, Menlo
Park, CA 95025 (US). DIAMOND, Jennifer; 421 Elder
Avenue, Millbrae, CA 94030 (US). MATHER, Christine;
1410 Millbrae Avenue, #203, Millbrae, CA 94030 (US).
BEEMER, Kathleen; 654 Hickey Blvd., Pacifica, CA
94044 (US). MYERS, Heather; 191 Sonora Avenue,
Danville, CA 94526 (US).

(74) Agents: MULVILLE, Kurt et al.; Orrick Herrington &
Sutcliffe LLP, 4 Park Plaza, Suite 1600, Irvine, CA 92614-
2558 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC,
VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii)) for all designations
- as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii)) for all designations

Published:

- with international search report

(88) Date of publication of the international search report:
4 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CHIMERIC BIRD FROM EMBRYONIC STEM CELLS

(57) Abstract: Sustained cultures of avian embryonic stem cells are provided. Injection of avian embryonic stem cells into recipient embryos yields chimeras with a significant contribution from the embryonic stem cell phenotype. Transgene encoding exogenous proteins are stably integrated in the embryonic stem cells and are present in the somatic tissue of the resulting chimeras. The transgenes may encode exogenous proteins expressed in endodermal, ectodermal, mesodermal, or extra embryonic tissue. Breeding the resulting chimera yields transgenic birds whose genome is comprised of exogenous DNA.

WO 03/064627 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/03235

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01K 67/00, 67/027; C12N 15/00, 5/00, 5/06, 15/63

US CL : 800/13, 19, 24; 435/320.1, 325, 349, 455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/13, 19, 24; 435/320.1, 325, 349, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,515,199 B1 (PETITTE et al) 04 February 2003, see entire document.	1-18
Y	US 6,333,192 B1 (PETITTE et al) 25 December 2001, see columns 11-14.	1-18
Y	US 5,830,510 A (PETITTE et al) 03 November 1998, see column 8.	1-18
Y	US 6,156,569 A (PONCE DE LEON et al) 05 December 2000, see column 8-9 and 12.	1-18
A	US 5,897,998 A (SPEKSNIJDER et al) 27 April 1999, see entire document.	1-18
Y	US 5,162,215 A (BOSELNAN et al) 10 November 1992, see column 9-10.	1-18
X	WO 00/47717 A1 (HANMI PHARM. CO. LTD.) 17 August 2000, see page 4, lines 10-31, page 8, line 30, page 14 line 5, page 15 line 34.	1-3, 9-11, 14-18
Y		4-8, 12-13

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier application or patent published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	*A* document member of the same patent family

Date of the actual completion of the international search

16 May 2003 (16.05.2003)

Date of mailing of the international search report

08 AUG 2003

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (703)305-3230

Authorized officer

Sumesh Kaushal Ph.D.

Telephone No. 703-308-0196

INTERNATIONAL SEARCH REPORT

PCT/US03/03235

Continuation of B. FIELDS SEARCHED Item 3:

STN-EXP: medline, biosis, agricola, scisearch, caplus.EAST-USPTO: pgpub, jpo, epo, derwent.

Search Terms: chicken, embryonic stem cell, transgenic, embryo, chimeric, chimera, transgene, transfect, transduce.